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Recombinant Human Bone Morphogenetic Protein 2B Stimulates PC12 Cell Differentiation: Potentiation and Binding to Type IV Collagen

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Abstract. Bone morphogenetic protein 2B (BMP 2B, also known as BMP 4) induces cartilage and bone morphogenesis in ectopic extraskeletal sites. BMP 2B is one of several bone morphogenetic proteins which along with activins and inhibins are members of the transforming growth factor-β (TGF-β) family. Both BMP 2B and activin A, but not TGF-β_1, induce rat pheochromocytoma PC12 neuronal cell differentiation and expression of VGF, a nervous system-specific mRNA. PC12 cells exhibited ~2,500 receptors per cell for BMP 2B with an apparent dissociation constant of 19 pM. Extracellular matrix components, including fibronectin, laminin, and collagen type IV potentiated the activity of BMP and activin A, with the latter being the most active. Direct experiments demonstrated that radioiodinated BMP 2B bound to collagen type IV better than to either laminin or fibronectin. These data demonstrate a common neurotrophic activity of both BMP 2B and activin A, and suggest that these regulatory molecules alone and in conjunction with extracellular matrix components may play a role in both the development and repair of nervous tissue.

GROWTH and differentiation factors in bone enable demineralized bone matrix to induce endochondral bone formation at ectopic sites in rat (Reddi and Huggins, 1972). Such bone-inducing factors have been extracted dissociatively from demineralized bone matrix by chaotropic reagents and reconstituted to demonstrate biological activity (Sampath and Reddi, 1981). Six different bone morphogenetic factors (BMP 2-7)[1] have been identified (Wozney et al., 1988; Luyten et al., 1989). The BMPs are members of a family of growth and differentiation polypeptides that includes activins A and B, inhibins, transforming growth factor-β (TGF-β), the Drosophila decapentaplegic gene complex, Mullerian inhibiting substance (for review see Massague, 1990), and growth and differentiation factor-1 (Lee, 1990). The different members of this superfamily have been implicated in various aspects of embryonic development. For example, Osteogenin/BMP 3, BMP 2, BMP 2B (also known as BMP 4), and osteogenic protein (BMP 7) induce endochondral bone induction in ectopic sites (Luyten et al., 1989; Ozkaynak et al., 1990; Hammonds et al., 1991; Wozney et al., 1988). Osteogenin/BMP 3 stimulates both alkaline phosphatase activity and collagen synthesis in rat periosteal cells and in calvarial osteoblasts (Vukicevic et al., 1989). Activins have been shown to stimulate secretion of follicle stimulating hormone and are important in neuronal survival (Vale et al., 1986; Schubert and Kimura, 1991). Activins have also been implicated in mesoderm induction in amphibians (Thomsen et al., 1990).

The activity of growth and differentiation factors can be modulated by components of the extracellular matrix (Paralkar et al., 1990; Ruoslahti and Yamaguchi, 1991). For example, heparin and heparan sulfate modulate the activity of fibroblast growth factors (Yayon et al., 1991). Many other growth and differentiation factors, which include neurite-promoting factor, pleiotropin, and granulocyte-macrophage colony-stimulating factor, have been shown to bind heparin and heparan sulfate present in the extracellular matrix molecules (Ruoslahti and Yamaguchi, 1991). Extracellular matrix macromolecules are also known to contain growth factor-like sequences and can induce the differentiation of various cell types. For example, laminin and entactin contain EGF-like domains, and laminin can induce neuronal cell lines and primary neurons to extend processes (Baron van Evercooren et al., 1982; Engel, 1989; Durkin et al., 1988; Reh et al., 1987). Another basement membrane component, collagen type IV, affects the attachment, spreading, and migration of various cell types (Herbst et al., 1988; Aumailley and Timpl, 1986). Recent work has also strongly implicated collagen type IV in neurite outgrowth (Lein et al., 1991; Mirre et al., 1992). Osteogenin/BMP 3 and TGF-β bind to collagen type IV which is present in basement membrane (Paralkar et al., 1990, 1991*b*).

We report here that BMP 2B has neurotrophic activity and induces the differentiation of rat pheochromocytoma (PC12) cells. The neurotrophic effects of BMP 2B are shared to a lesser extent by activin A but not by TGF-β_1. We also show

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1. *Abbreviations used in this paper*: BMP, bone morphogenetic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NGF, nerve growth factor; TGF, transforming growth factor.

that extracellular matrix molecules can potentiate the activity of BMP 2B with type IV collagen being the most active.

Materials and Methods

Materials

Recombinant human BP 2B (rBMP 2B), TGF-β_1, and activin A were gifts of Dr. Glenn Hammonds (Genentech Inc., San Francisco, CA). Basic FGF, and the AA, BB, AB isoforms of PDGF were kindly provided by Dr. Glenn Pierce (Amgen Biologicals, Thousand Oaks, CA). Nerve growth factor (NGF) was purchased from Sigma Chem. Co., St. Louis, MO. The plasmid containing the VGF probe was a gift of Dr. Bruce Patterson (National Cancer Institute). Collagen type IV and laminin were prepared from the murine EHS tumor as previously described (Kleinman et al., 1982). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was from American Type Culture Collection, Rockville, MD (No. 57090).

Cell Culture and Neurite Outgrowth Assay

The PC12 cells (a gift of Dr. G. Guroff, National Institute of Child Health and Human Development) were grown in DME containing 7.5% FCS and 7.5% horse serum. For initial assessment, PC12 cells were cultured in 100-mm tissue culture plates in DME containing serum. Various growth factors such as NGF, FGF, PDGF isoforms AA, AB, and BB, BMP 2B, activin A, and TGF-β were then added at a final concentration of 0.1–50 ng/ml. After 48 h, the cells were photographed using ASA400 film.

For neurite outgrowth assays, PC12 cells were washed extensively in serum-free DME/Ham's F12 (1:1) containing 100 μg/ml transferrin, 100 μm putrescine, 20 nM progesterone, 30 mM $NaSeO_3$, and 5 μg/ml insulin. Cells (10,000/0.5 ml) were then plated in a 24-well tissue culture plate where the wells had been either untreated or coated with either 10 μg laminin, poly-L-lysine, fibronectin, or collagen type IV in PBS for 1 h at 37°C. NGF, activin A, or BMP 2B were added at the indicated concentrations (see figure legends) and process formation was quantitated after 24 h. Cells were considered positive for formation if the process length equaled or was greater than one cell diameter. A total of 100 cells were scored per data point. For photomicrographs, the cells were fixed and stained with Diff Quick (American Scientific Products Division, McGaw Park, IL). In separate experiments, the effect of a bacterial alkaloid K-252a on the ability of both activin A and BMP 2B to stimulate PC12 cell neurite outgrowth was tested. PC12 cells were cultured on 16-mm-diam wells which had been coated with 0.1 μg/well of type IV collagen. Different growth factors (50 ng/ml) were added along with K-252a at the time of cell seeding and then the cells were fixed after 24 h of incubation. The stock solution of K-252a was prepared in 100% DMSO and diluted in DME for use. DMSO alone at the concentration used had no effect on PC12 cells.

For cell attachment, collagen type IV, laminin, polylysine, and fibronectin were coated as described for neurite outgrowth assays, but after coating, each well was incubated at 37°C for 1 h with a solution of 3% BSA. Cells (50,000/well) were plated in the presence or absence of BMP 2B (50 ng/ml). After 1 h, unattached cells were removed by aspiration and attached cells were fixed and stained with Diff Quick. The number of cells was counted in three fields at 10× and the mean ± standard error is presented.

Iodination of BMP 2B and Binding Assay

PC12 cells were examined for their ability to bind radioactive BMP 2B in a specific and saturable manner. Recombinant BMP 2B was iodinated by the chloramine T method as described previously (Paralkar et al., 1991*a*). For Scatchard analysis, PC12 cells were washed three times with DME containing 1 mg/ml BSA and 20 mM Hepes (binding medium). The cells were then incubated in binding medium. After 1 h, the cells were centrifuged and resuspended at a final density of 3×10^6/ml in binding medium. 100 μl of the cell suspension was then mixed with 100 μl of binding medium containing increasing concentrations of I^{125}-labeled BMP 2B in a siliconized microfuge tube. After incubation with radiolabeled BMP 2B for 1 h at room temperature, 1 ml of ice cold binding buffer was added to each tube and the tubes were centrifuged at 12,000 *g* for 2 min. 600 μl of buffer from each tube was then removed and the radioactivity was determined in a gamma counter (Beckman Instrs., Inc., Fullerton, CA). The remaining medium was then removed and the cell pellet was resuspended in 100 μl of binding buffer. The resuspended cell pellet was then layered on top of a 200-μl oil parafin mixture in a 400-μl microfuge tube. The tubes were then centrifuged at 12,000 *g* for 2 min. At the end of centrifugation, the supernate was removed and the bottom of the tube containing the cell pellet was cut with a razor blade and the radioactivity was determined in a gamma counter. Each time point was determined in triplicate. Nonspecific binding was determined by using a 100-fold excess of unlabeled BMP 2B.

The binding of iodinated BMP 2B to extracellular matrix macromolecules was determined as previously described (Paralkar et al., 1990). Briefly, the various macromolecules were applied to a nitrocellulose membrane in a final volume of 100 μl. The membrane was first blocked by incubation with 3% BSA in PBS containing 0.05% Tween 20 (TPBS), and then incubated with radioactive BMP 2B in TPBS. After incubation with BMP 2B, the membrane was washed and then exposed to x-ray film for autoradiography.

Detection of VGF Synthesis

100-mm tissue culture plates were coated either with collagen type I or collagen type IV. PC12 cells extensively washed with serum-free DME were then seeded (5×10^6 per plate) in serum-free DME and treated with either NGF, activin A, or BMP 2B (50 ng/ml) for 5 h. Control cells did not receive any growth factor. After incubation, total RNA was prepared from the cells as described (Chomczynski and Saachi, 1987). Approximately 10 μg of total RNA from each treatment was subjected to electrophoresis on agarose-formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, NH). The blot was hybridized with ^{32}P-labeled VGF fragment (EcoI, HindIII) and ^{32}P-labeled human GAPDH probe. The amount of induction of VGF, a nervous system-specific transcript, was quantified by scanning the autoradiographs with a densitometer using the amount of GAPDH hybridized for normalization.

Results

Neurite Outgrowth in Response to BMP 2B and to Activin A

We studied the effect of BMP 2B and activin A on rat pheochromocytoma PC12 cells. BMP 2B and activin A stimulated neurite outgrowth in PC12 cells (Fig. 1). The specificity and magnitude of the response of PC12 cells were tested using several other polypeptide growth factors including members of the TGF-β superfamily such as activin A and TGF-β_1. Table I shows that of the factors tested, NGF (positive control), basic FGF, BMP 2B, and activin A had neurotrophic activity for PC12 cells whereas TGF-β_1 or the various isoforms of platelet-derived growth factor (AA, AB, and BB) had no neurotrophic activity. Activin has been previously shown to be a neuronal cell survival molecule (Schubert et al., 1990). Activin A can also induce neurite outgrowth in PC12 cells but was not as active as BMP 2B or NGF. In these experiments, the different growth factors were tested in the presence of serum. We next determined if the effect of both BMP 2B and activin A was demonstrable in the absence of serum components. Both BMP 2B and activin A were ineffective in the absence of serum (data not shown).

Effect of Extracellular Matrix Macromolecules

Since many growth factors act in combination with extracellular matrix components, the effects of extracellular matrix macromolecules on the neurotrophic activity of both BMP 2B and activin A were tested. When the matrix components were present at a substratum, activity was observed in serum-free conditions. NGF has been shown to be a potent stimulator of neurite outgrowth especially in combination with laminin and was used as a positive control. Like NGF, BMP 2B and activin A had higher neurotrophic activity when the cells were plated on either fibronectin, laminin, or

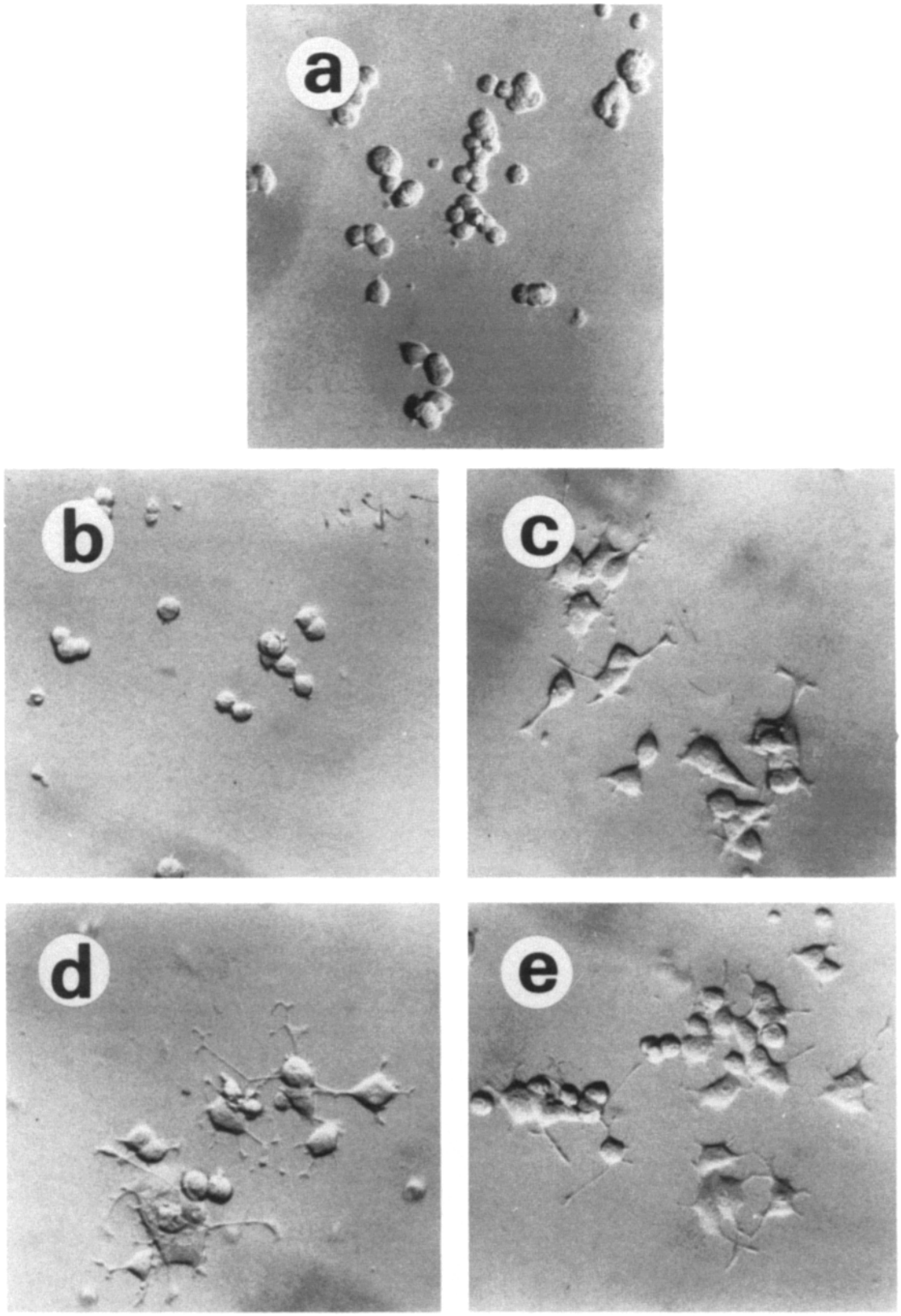

Figure 1. Induction of neurite outgrowth in PC12 cells treated with various growth factors. PC12 cells were cultured in 100-mm tissue culture dishes in the presence of medium containing serum. Different growth factors at 50 ng/ml were then added and the cells were fixed after 48 h. (*a*) Control PC12 cells, (*b*) PC12 cells with TGF-β_1, (*c*) PC12 cells with activin A, (*d*) PC12 cells with NGF, and (*e*) PC12 cells with BMP 2B.

Table I. Effect of Growth Factors on Neurite Extension in PC12 Cells in the Presence of Serum

Growth factor	Neurite extension
NGF	+++
Basic FGF	+
BMP2B	++
Activin A	+
TGF-β_1	−
PDGF AA	−
PDGF BB	−
PDGF AB	−
IGF-1	−

+++ corresponds to 70–100% of the cells; ++ corresponds to 40–70% of the cells; and + corresponds to 40% of the cells having significant processes. − signifies lack of neurite extension. Cells were treated with the indicated growth factors for 48 h in the presence of serum. Neurite process formation was enumerated when the process length was greater than one cell diameter. The experiment was repeated two times and each factor was tested in triplicates.

collagen type IV than when tested on poly-L-lysine–coated plastic or on plastic alone (Fig. 2). Poly-L-lysine is highly adhesive for the cells used and was used as a control to exclude adhesion effects from the differentiation activity. Poly-L-lysine did not stimulate neurite outgrowth in the presence or absence of growth factors but was as active as laminin and collagen type IV for cell attachment. Laminin and collagen

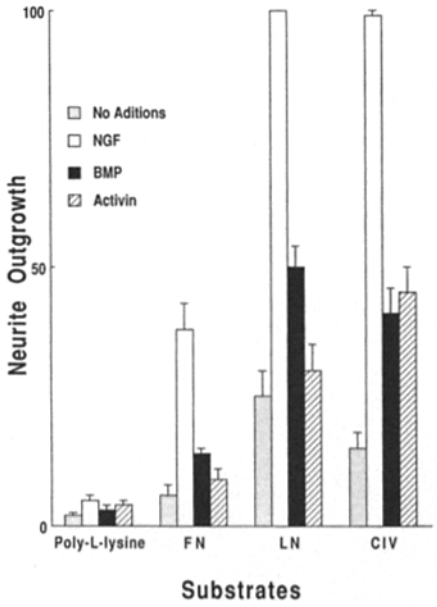

Figure 2. Effect of BMP 2B and activin A on neurite outgrowth in the presence of extracellular matrix components. PC12 cells were cultured in duplicate 16-mm-diam wells which had been coated with 10 μm collagen type IV, laminin, fibronectin, or poly-L-lysine. Cells were grown in uncoated plates and primed for 24 h with 50 ng/ml of BMP 2B, activin A, or NGF. After 24 h, cells were harvested, washed free of serum, and then transferred to plates precoated with extracellular matrix macromolecules. Growth factors (50 ng/ml) were added and the cells were cultured for an additional 24 h in the absence of serum. At the end of incubation, 100 cells were counted in two fields/well for neurite outgrowth activity. Cells with processes greater than one cell diameter were considered positive. The number of positive cells was enumerated and presented as the mean ± SEM.

Table II. Attachment of PC12 Cells to Polylysine and to Extracellular Matrix Macromolecules

	Cells/field*	
Substratum	No addition	BMP 2B
Fibronectin	44 ± 6	54 ± 4
Laminin	104 ± 4	116 ± 14
Collagen type IV	130 ± 4	139 ± 8
Poly-L-lysine	124 ± 10	118 ± 6
Control plastic	10 ± 2	6 ± 2

Cells were plated on plastic wells coated with various extracellular molecules in the presence (50 ng/ml) or absence of BMP 2B.
* The results are mean ± SE of three different fields.

type IV (10 μg/well) were approximately equally active and more active than fibronectin in potentiating the effect of both BMP 2B and activin A. These differences could not be attributed to attachment alone since attachment to laminin, collagen type IV, and poly-L-lysine was identical and was not affected by BMP 2B treatment (Table II). Attachment to fibronectin was less than to laminin, collagen type IV, and poly-L-lysine, which may explain in part the reduced activity of fibronectin. When varying doses of collagen type IV and laminin were used, BMP 2B was more active at lower doses of collagen type IV (0.001–1 μg/well) when compared to laminin at the same levels (Fig. 3). At higher amounts, both laminin and collagen type IV were equally active in potentiating the effects of BMP 2B.

Since BMP 2B and activin A promoted PC12 cell differentiation, the mechanism of action was investigated. K-252a, a bacterial alkaloid, is an inhibitor of NGF-induced neurite extension in PC12 cells but does not affect FGF-induced neurite extension (Koizumi et al., 1988; Weeks et al., 1990). K-252a inhibited the effects of NGF, activin A, and BMP 2B by 84, 42, and 95%, respectively, on a collagen IV substratum. Thus, both BMP 2B and activin A likely stimulate a second messenger pathway common to that elicited by NGF.

Binding of Radiolabeled BMP 2B to Extracellular Matrix Macromolecules

Since collagen type IV potentiated the activity of BMP 2B better than laminin, and osteogenin/BMP 3 is known to bind collagen type IV (Paralkar et al., 1990), collagen type IV binding to iodinated BMP 2B was tested. Iodinated BMP 2B bound to collagen type IV preferentially over laminin (Fig. 3 *B*). No binding was observed for other collagens tested including collagen II, IX, and XI; and relatively weak binding was observed to collagen I. Matrigel which is composed of laminin (70%) and collagen IV (5%) also had some binding. Thus, BMP 2B appears to specifically interact with collagen IV over other matrix proteins.

Dose Response of BMP 2B, Activin A and NGF

The effect of BMP 2B, activin A, and NGF on collagen type IV was concentration dependent (Fig. 4). BMP 2B had ~50% of the activity of NGF. The concentration of all three factors at half-maximal response is very similar (0.3–0.6 nM) but it should be noted that the maximal responses plateau at different levels. The difference between the effect of NGF and BMP 2B increases as the concentration increases

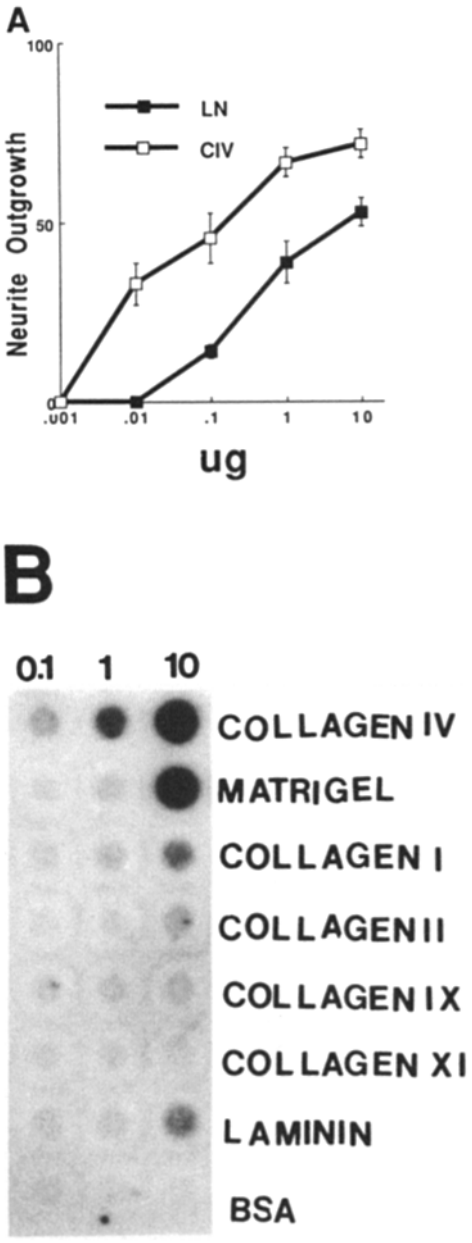

Figure 3. (*A*) Neurite outgrowth promoting activity of BMP 2B with cells cultured on various amounts of collagen type IV and laminin. BMP 2B was added at 50 ng/ml at the time of PC12 cell seeding into 16-mm-diam wells which had been coated with the indicated amounts of either collagen type IV or laminin. Duplicate wells were prepared and 100 cells/well were counted in the center field of each well. The number of cells with processes 1× the cell diameter or greater is presented as the mean ± SD. (*B*) Collagen types I, II, IV, IX, and XI, laminin, Matrigel, and BSA were immobilized on nitrocellulose at three different concentrations (0.1, 1, and 10 μg). The nitrocellulose membrane was then incubated with radioactive BMP 2B, washed, and exposed to x-ray film for autoradiography.

(above 1 nM). Maximum response to BMP 2B is ~1 nM whereas the maximum response to NGF is seen at ~2.5 nM.

Demonstration of Specific ^{125}I-BMP 2B Binding to PC12 Cells

Radiolabeled BMP 2B bound in a specific and saturable manner to rat pheochromocytoma cells with a dissociation constant of 19 pM with 2,500 receptors per cell (Fig. 5). A low number of high affinity sites has been demonstrated for BMP 2B in other cell types (Paralkar et al., 1991*a*). PC12 cells bound radiolabeled NGF in a specific and saturable manner (data not shown). NGF did not compete for BMP 2B.

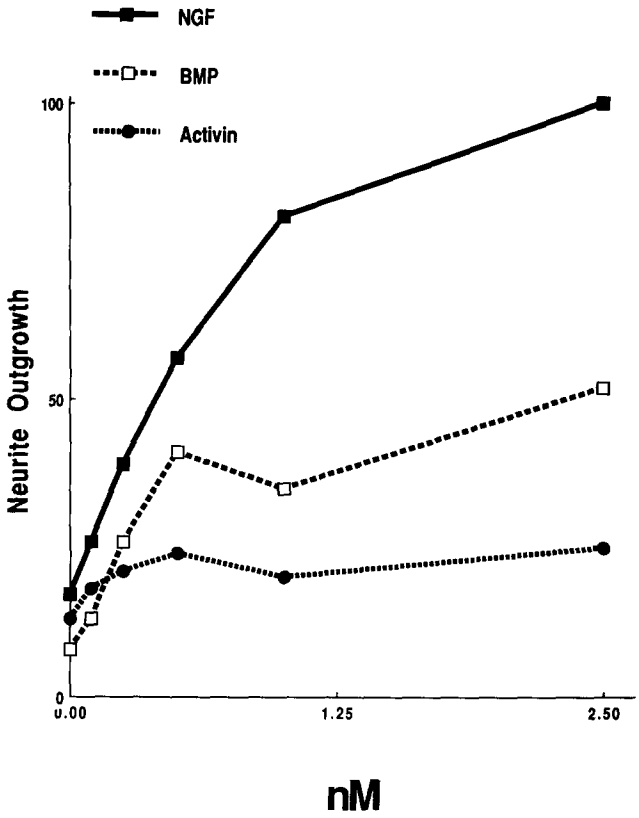

Figure 4. Effect of equimolar doses of NGF, BMP 2B, and activin A on neurite outgrowth. PC12 cells were cultured on 16-mm-diam wells which had been coated with 0.1 μg/well of collagen type IV. The growth factors were added at the time of cell seeding and then the cells were fixed after 24 h of incubation. 100 cells were counted in the center field of each well at 10× and cells with process length 1× the cell diameter were considered positive.

Regulation of VGF mRNA Levels in PC12 Cells

We examined the induction of a nervous system–specific VGF mRNA expression to verify the state of differentiation of PC12 cells. Cells were grown on plates coated with either collagen type I or collagen type IV. Northern analysis of transcripts showed a rapid induction of VGF mRNA when cells were treated with either NGF, BMP 2B, or activin A (Fig. 6). The induction was maximum for NGF (seven- and fourfold over control for cells grown on collagen types I and IV, respectively) but was also significant for both activin A (five- and threefold) and BMP 2B (seven- and threefold over control).

Discussion

This report establishes that BMP 2B, a bone morphogenetic protein, stimulates neurite extension in rat pheochromocytoma PC12 cells via functional receptors for BMP 2B. These

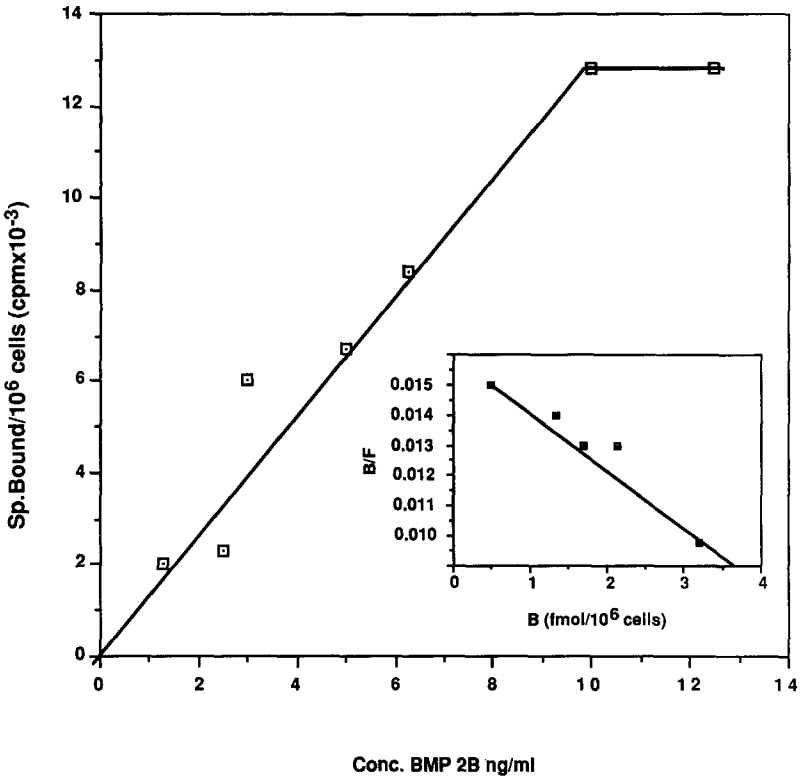

Figure 5. Binding of ^{125}I BMP 2B to PC12 cells. PC12 cells were incubated with increasing concentrations of BMP 2B at room temperature for 1 h. Specifically bound radioactivity was determined by subtracting nonspecific binding from total cell-bound radioactivity for each data point. (*Inset*) Scatchard analysis of the binding data. *B*, bound; *F*, free.

effects of BMP 2B on PC12 cells are similar to those demonstrated for NGF and were also exhibited by activin A. In addition, activin has been demonstrated to be involved in mesoderm induction in *Xenopus* (Thomsen et al., 1990). In serum-free conditions in vitro, the effect of all three factors is dependent on the extracellular matrix component used to coat the tissue culture wells. Collagen type IV was the most potent extracellular matrix macromolecule tested. The differences in activity of the matrix molecules used to coat the wells cannot be explained on the basis of differences in cell attachment, since poly-L-lysine which is as potent as collagen type IV in promoting cell attachment does not stimulate neurite outgrowth in the presence or absence of BMP 2B. We have previously shown that purified bovine osteogenin/BMP 3 and TGF-β_1 and TGF-β_2 bind to collagen type IV, and that collagen type IV can modulate the activity of TGF-β (Paralkar et al., 1990; Paralkar et al., 1991*b*). Radiolabeled recombinant human BMP 2B also has an affinity for collagen type IV preferentially over that observed for laminin. The observation that collagen type IV had a similar ability to promote the activity of all three growth and differentiation factors suggests a possible common pathway for the action of BMP

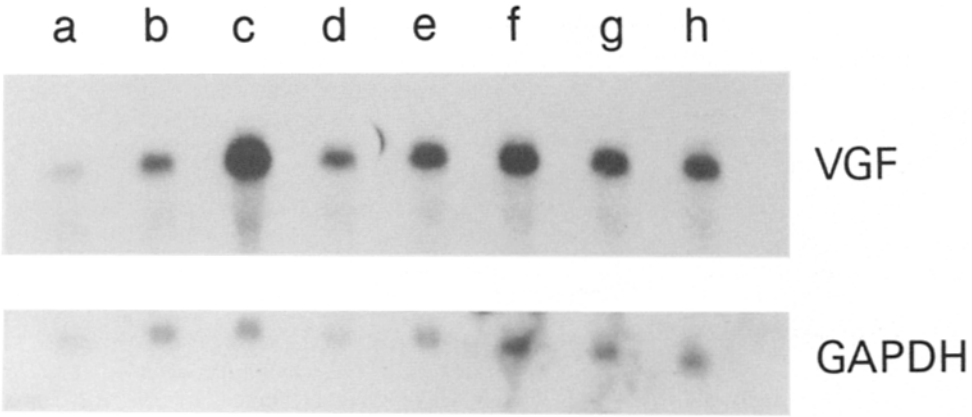

Figure 6. Induction of VGF mRNA synthesis by BMP 2B, NGF, and activin A. Northern blot analysis of mRNA from PC12 cells grown on collagen type I (lanes *a*, *c*, *e*, and *g*) or IV (lanes *b*, *d*, *f*, and *h*) and treated with NGF, BMP 2B, or activin A. Control wells were not treated with any growth factor. RNA (15 μg) was separated in 1% agarose gel containing formaldehyde, and immobilized on Nytran. The autoradiograph shows results of hybridizations with VGF and GAPDH probes. Lanes *a* and *b* are control, *c* and *d* were treated with NGF, *e* and *f* with BMP 2B, and *g* and *h* with activin A. The autoradiograph was scanned by densitometry.

2B, NGF, and activin A. Further similarities were seen by the ability of both activin A and BMP 2B to stimulate gene expression of VGF and the ability of K-252a to inhibit the activity of both BMP 2B and activin A. The precise mechanism of action of all three factors is unknown. The activin receptor is a membrane-spanning serine/threonine protein kinase (Mathews and Vale, 1991) whereas the NGF receptor is a protein tyrosine kinase (Kaplan et al., 1991).

Much is known about the role of extracellular matrix macromolecules in tissue repair, cell attachment, and differentiation (Reddi, 1984; Engel, 1989; Kleinman et al., 1990). Numerous other functions have been ascribed to the extracellular matrix macromolecules. Laminin, for example, has been shown to be able to differentiate epithelial cells in vitro (Reh et al., 1987). Attention has been focused on extracellular matrix macromolecules because of their ability to bind growth and differentiation factors (Folkman et al., 1988; Paralkar et al., 1990; 1991*b*; Ruoslahti and Yamaguchi, 1991). The extracellular matrix macromolecules might play an important physiological role in sequestration and modulation of activity of growth and differentiation factors. These interactions of growth and differentiation factors with extracellular matrix macromolecules may result in the localization of a bioactive molecule at the appropriate site in vivo to facilitate local action by preventing diffusion of soluble growth factors. There are numerous advantages in the interaction of extracellular matrix macromolecules with growth and differentiation factors, such as protection from proteolytic degradation, availability of high concentration of active molecules at the appropriate site, and minimizing the rate of diffusion. This investigation demonstrates that collagen type IV not only binds BMP 2B but also modulates the activity of BMP 2B on PC12 cells.

The in vivo role of different BMPs for functions other than bone induction has not yet been demonstrated. The expression pattern of BMP 2A mRNA in various tissues, including brain, by in situ hybridization lends further support to the physiological significance of the present study (Lyons et al., 1990). There are now six different BMPs (BMP 2 through 7) present in bone which can induce bone at ectopic sites in the in vivo bioassay. Although these BMPs were isolated by in vivo bone morphogenetic bioassay, it is possible that some of these BMPs might be involved in embryonic development and repair of other tissues. Indeed this possibility is supported by the present results demonstrating the neurotrophic activity of BMP 2B. In conclusion, recombinant human bone morphogenetic protein 2B is a neurotrophic molecule and its activity is increased by collagen type IV.

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